Preparation of Agaro-oligosaccharides

Agar was resuspended in 0.1N HCl to 10% (w/v) and heated at 100°C for 15 min. After centrifuging the sample to remove the insolubles, the Agaro-oligosaccharide solution was subjected to gel filtration chromatography in Toyopearl HW-40C (45 mm × 100 cm, Toso) to obtain Agaro-oligosaccharides of various lengths. The gel filtration chromatography was conducted at the rate of 1 mL/min and pure water was used as the carrier. The various fractions were analyzed by thin layer chromatography (TLC) using silica gel 60 F_{254} and butanol : ethanol : water (5 : 3 : 3) as the solvent system. The TLC analysis results indicated that various Agaro-oligosaccharide fractions were obtained, so that Agarobiose, Agarotetraose and Agarohexaose were prepared.

Cell culture of RAW264.7

The mouse macrophage cell line RAW264.7 (ATCC TIB71) was obtained from Dainippon Pharmaceutical Co., Ltd. The cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) containing 10% fetal bovine serum (JRH Biosciences) and antibiotics (50 units/mL penicillin, 50 μg/mL streptomycin, Gibco BRL). The cells were passaged every 3 days. For the evaluation of NO synthesis, the cells were suspended in culture medium to give 4×10^5 cells/mL and placed in 48-well plates at 0.5 mL. For evaluation of HO-1 induction, the cells were suspended in culture medium to give 3×10^5 cells/mL and placed in 6-well plates at 5 mL. After overnight culture, fresh medium was added, and the assays were conducted.

Evaluation of HO-1 induction

To the RAW264.7 cells, various concentrations of Agaro-oligosaccharide solutions were added and cultured for 12 hr. At the end of the culture period, the cells were collected, washed in PBS, resuspended in lysis buffer (0.1% Triton X-100, 10 mM EDTA-2Na, 1 mM PMSF, 0.2 mM leupeptin, 0.05 mM pepstatin A in 100 mM Tris HCl, pH 7.4), and subjected to one cycle of freeze-thaw to prepare the cell extract. This was then centrifuged at 4°C, 10,000 rpm, for 20 min, and the supernatant was collected. The protein concentration in the samples was measured using the Micro BCA protein assay reagent (Pierce Chemical Company). Cell extract containing 10 μg of the protein was mixed with the Laemmli loading buffer, heated at 100°C for 5 min, and applied to 12.5% polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membrane (Millipore Japan). The signal was detected by chemiluminescence (Renaissance, NEN Life Science Products) using anti-HO-1 polyclonal antibody (Santa Cruz Biotechnology), with anti-α tubulin monoclonal antibody (Calbiochem) as the

internal control.

Evaluation of NO synthesis

Agarobiose was added to RAW264.7 cells at various concentrations and cultured for 5 hr. LPS was then added to a final concentration of 1 μg/mL and IFN-α to final concentration of 10 U/mL and cultured for 16 hr. After the incubation period, the culture medium was assayed for nitrite formed as a result of NO hydrolysis as an index of NO synthesized. 100 μL of the culture supernatant was treated with 100 μL of the 4% Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl-)ethylene diamine in 5% o-phosphoric acid, Sigma) and measured after 15 min in a plate reader at 540 nm absorbance.

Isolation and storage of human peripheral blood mononuclear cells (PBMC)

400 mL of blood was obtained from human healthy donors. The blood was diluted 2 fold in PBS(-), overlaid on Ficoll-paque (Pharmacia) and centrifuged for 20 min at 500 × g. The peripheral blood mononuclear cells (PBMC) at the interphase were collected with a pipette and washed in RPMI1640 medium (BioWhittaker). The PBMC collected was resuspended in the freezing solution of 90% FCS (JRH Biosciences)/10% dimethyl sulfoxide, and stored in liquid nitrogen. At the time of the experiment, the PBMC stored was rapidly thawed in a 37°C water bath, washed in RPMI1640 medium containing 10 μg/mL DNase (Calbiochem), and cell viability tested by the trypan blue dye exclusion method.

Isolation of human PBMC derived monocytes

The PBMC was suspended at 2 × 10^6 cells/mL in RPMI 1640 medium containing 5% human AB serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine (all from BioWhittaker), 10 mM HEPES and antibiotics (50 units/mL penicillin, 50 μg/mL streptomycin, Gibco BRL) (5H RPMI medium), plated in 24-well plates at 1 mL/well, and incubated for 1.5 hr. At the end of 1.5 hr, the non-adherent cells were removed, and each well was washed with RPMI 1640, and then 1 mL of 5H RPMI medium added to obtain human monocytes.

Evaluation of production of PGE₂ and various cytokines

Agarobiose was added at various concentrations to the human monocytes and cultured for 5 hr. LPS was then added to the final concentration of 1 ng/mL, and culture continued for 16 hr for PGE_2 measurements or for 4 hr for measurements of

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TNF- α, IL-1 β and IL-6. The culture supernatants were collected and the PGE$_2$, TNF- α, IL-1 β and IL-6 contents determined using ELISA kits (PGE$_2$ (Neogen), TNF- α (Endogen), and IL-1 β and IL-6 (Genzyme Techne)).

Results

Induction of Heme oxygenase-1 by Agaro-oligosaccharide

Induction of Heme oxygenase-1 by Agaro-oligosaccharide was studied in the mouse macrophage cell line RAW264.7 cells. As indicated in Figure 1, with medium alone, RAW264.7 cells at 12 hr later showed no detectable levels of HO-1. In contrast, the addition of Agaro-oligosaccharides resulted in a dramatic induction of HO-1. This HO-1 induction was seen with all Agaro-oligosaccharides, Agarobiose, Agarotetraose and Agarohexaose, and the expression level increased in a dose dependent manner. On the other hand, neo-Agarobiose, which has a galactose at the reducing end, did not induce HO-1 expression. The HO-1 induction by Agaro-oligosaccharides was determined to be at the transcriptional level by RT-PCR. It was also determined that HO-1 induction occurred at the transcriptional level in human PBMC derived monocytes (data not shown).

Studies on inhibition of NO production by Agarobiose

Using the RAW264.7 cells we studied the effect of Agarobiose on nitrite accumulation after LPS or IFN- γ stimulation. As an index of NO synthesis by cells, we measured the accumulation of nitrite produced by hydrolysis of NO by water present in the culture supernatant using the Griess reagent. Using this system, we determined that even a high concentration of Agarobiose (1 mM) had no effect on the reaction of nitrite with the Griess reagent (data not shown). Nitrite production by RAW264.7 was dependent on the activation state. Activation by LPS or IFN- γ resulted in a 90-fold increase in the nitrite (about 31 μM) compared to the non-stimulated state (about 0.37 μM). In contrast, the addition of Agarobiose prior to the stimulation resulted in a dose-dependent inhibition of NO synthesis, with about 50% inhibition at 100 μM of Agarobiose (Figure 2). Similar results were obtained with Agarotetraose and Agarohexaose, while neo-Agarobiose gave no inhibition (data not shown). In this experimental system, Agaro-oligosaccharides had no cellular toxicity even at high concentrations as determined by the MTT assay (data not shown).

Assessment on inhibition of PGE$_2$ and pro-inflammatory cytokine production by agarobiose

Effect of Agarobiose on LPS-stimulated production of PGE$_2$ and pro-inflammatory cytokines was studied in human PBMC derived monocytes. The assays were conducted using commercially available ELISA kits; with all kits it was determined that Agarobiose even at high concentrations (1 mM) had no effect on the reaction system (data not shown). The production of PGE$_2$ and various pro-inflammatory cytokines by human monocytes requires an activated state, and LPS stimulation results in an increase in PGE$_2$ by two fold (about 47 ng/mL) compared to the unstimulated state (about 23 ng/mL), an increase in TNF-α by about 60 fold (to about 3000 pg/mL) compared to the unstimulated state, an increase in IL-1β by about 6 fold (to about 240 pg/mL) compared to the unstimulated state (about 40 pg/mL), and increase in IL-6 by about 4 fold (to about 1200 pg/mL) compared to the unstimulated state (about 300 pg/mL). In contract, the addition of Agarobiose prior to stimulation resulted in dose-dependent inhibition of the production of PGE$_2$ and pro-inflammatory cytokines. The addition of 50 μM Agarobiose resulted in the inhibition of production of PGE$_2$ by about 75%, TNF- α by about 57%, IL-1 β by essentially 100%, and IL-6 by about 50% (Figure 3 (a) PGE$_2$; 3(b) TNF- α; 3(c) IL-1 β; 3(d) IL-6). In this experimental system, it was also confirmed that Agarobiose at high concentrations had no cellular toxicity by the MTT assay (data not shown).

Discussion

In this study, it was demonstrated that Agaro-oligosaccharides, oligosaccharides derived from a dietary fiber, inhibit the production of NO, PGE$_2$ and various pro-inflammatory cytokines by macrophages.

It has previously been thought that dietary fiber such as agar has no notable physiological activity and that any cancer preventive property may be attributable to physical effects such as improvement in stool transit. There have essentially been no studies conducted on physiological activity of dietary fiber and oligosaccharides. Specifically, inhibition of activated macrophages and anti-inflammatory effects by dietary fiber and oligosaccharides is to the best of our knowledge previously unreported.

Agaro-oligosaccharides, which are structural oligosaccharides derived agar, induced Heme oxygenase-1 in the mouse macrophage cell line RAW264.7 in a dose dependent manner. This induction was seen with Agaro-oligosaccharides having the 3,6-anhydro-L-galactose at the reducing end, such as Agarobiose, Agarotetraose and Agarohexaose, while neo-Agarobiose with D-galactose at the reducing end was inactive.

In the studies of inhibition of NO production in the same RAW264.7 cells, Agaro-oligosaccharides inhibited NO synthesis, but neo-Agarobiose had no activity. Therefore, the physiological activity of the Agaro-oligosaccharides appears to require 3,6-anhydro-L-galactose at the reducing end and that this is responsible for the induction of HO-1 and inhibition of NO production.

When HO-1 is induced, heme is degraded, releasing biliverdin, iron and CO[11]. The biliverdin is metabolized to bilirubin through the effects of biliverdin reductase. This bilirubin has attracted attention as a physiological compound with antioxidant properties involved in the elimination of activated oxygen species and prevention of lipid peroxidation[15,16,17]. Thus, at inflammatory sites it may remove activated oxygen species and prevent worsening of the lesions. Therefore, HO-1 induction by Agaro-oligosaccharide may inhibit the production of NO by macrophages and the production of activated oxygen by neutrophils that have infiltrated RA lesions and inhibit the injury directly. Recently, Choi et al., reported that the CO produced through the action of HO-1 inhibits the production of pro-inflammatory cytokines produced by activated macrophages such as TNF-α, IL-1β, MIP-1β and MIP-2[14]. Various cytokines have been implicated in RA. The Agaro-oligosaccharides induce HO-1, and the CO thus produced may inhibit the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, which have been therapeutic targets. In fact, Agarobiose inhibited production of these cytokines induced by LPS activation of human monocytes. This inhibitory effect is clearly observed when the Agarobiose is added to the monocytes prior to LPS stimulation. Therefore, it was suggested that the Agarobiose induces some sort of factor, resulting in the inhibition of the production of pro-inflammatory cytokines. Whether the Agarobiose directly inhibits all of these pro-inflammatory cytokines is currently unclear. However, since the effect is markedly seen when added prior to stimulation, the mechanisms of inhibition may be similar. Therefore, by adding Agarobiose prior to stimulation, it was suggested that HO-1 is sufficiently induced and CO is produced, thereby suppressing the pro-inflammatory cytokines.

Furthermore, Agarobiose inhibits the PGE$_2$ production by LPS-stimulated human monocytes. Anti-inflammatory agents such as aspirin have long been known to inhibit COX, which is a key enzyme in prostaglandin synthesis. COX exists as 2 isozymes, COX-1, which is expressed constitutively, and COX-2, which is induced at sites of inflammation by mitogenic stimuli[18,19]. Conventional non-steroidal anti-inflammatory agents (NSAIDs) inhibit both of these enzymes, and adverse effects are attributable to the inhibition of COX-1, which plays an important role in the protection of the gastric mucosa. Recently developed COX-2 selective NSAIDs inhibit only COX-2.

Agarobiose has no effect on PGE_2 production produced by COX-1 (data not shown), and its inhibitory effect involves a cascade that specifically includes COX-2. It has been shown that this is not due to COX-2 enzyme inhibition, but it has not been determined what step is inhibited in the PGE_2 production. PGE_2 synthase, which is downstream of COX-2, has been recently identified and cloned[20]. Since there have been no reports on the relationship between PGE_2 synthase and CO, further studies are needed to assess the possibility that Agarobiose inhibits the PGE_2 synthase.

Antibody therapeutic agents such as the anti-TNF antibody and anti-inflammatory agents such as NSAIDs are used in the treatment of RA. In the current study, we obtained data suggesting that Agaro-oligosaccharides, which are oligosaccharides derived from dietary fiber, may have preventive and therapeutic effects in RA. Three sites of action are thought to be possible in the prevention and treatment of RA by Agaro-oligosaccharides (Figure 4). Agaro-oligosaccharides induce HO-1, resulting in the synthesis of CO and bilirubin. This may cause (1) the inhibition of NO production by macrophages activated at the sites of inflammation, while the bilirubin may promote the elimination of activated oxygen, to inhibit tissue injury directly; (2) Selective inhibition of the COX-2 cascade in the PGE_2 production in activated macrophages, resulting in anti-inflammatory effects with no adverse effects; (3) inhibition by CO of the synthesis of pro-inflammatory cytokines by activated macrophages, so that induction of inflammation inhibited, resulting in therapeutic benefit.

Therefore, Agaro-oligosaccharides, which are oligosaccharides derived from agar, a dietary fiber used in Japan since ancient times, may be a previously unknown, new type of food product that may be useful in the prevention or treatment of RA at multiple sites of action.

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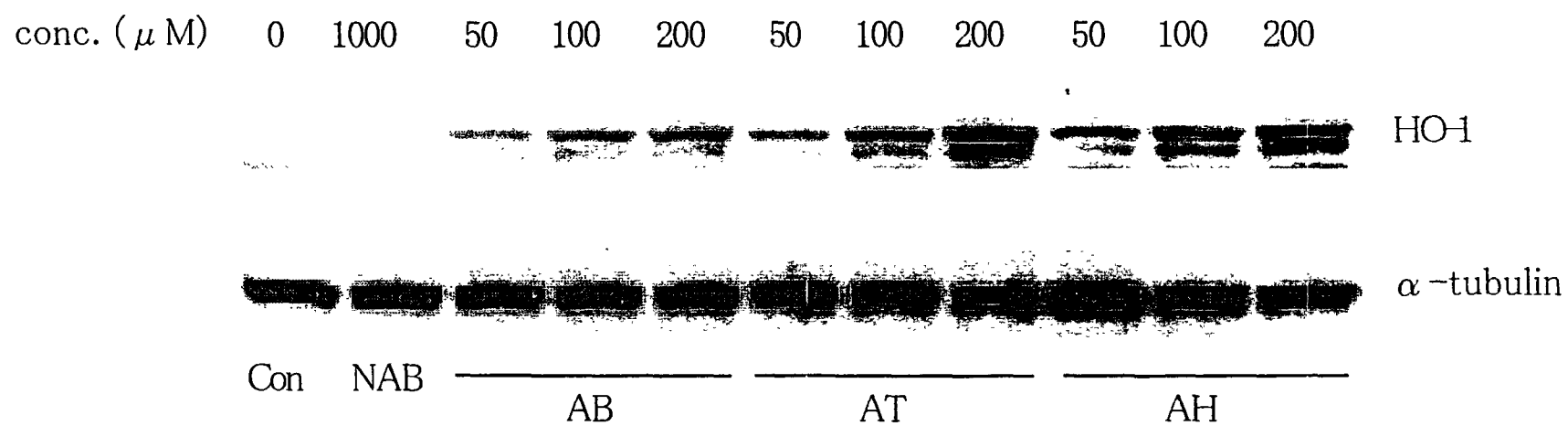
conc. (μM)   0   1000   50   100   200   50   100   200   50   100   200

HO-1

α-tubulin

Con   NAB ——————————— ——————————— ———————————
                    AB   AT   AH

Fig.1 Effects of various oligosaccharides on the levels of HO-1
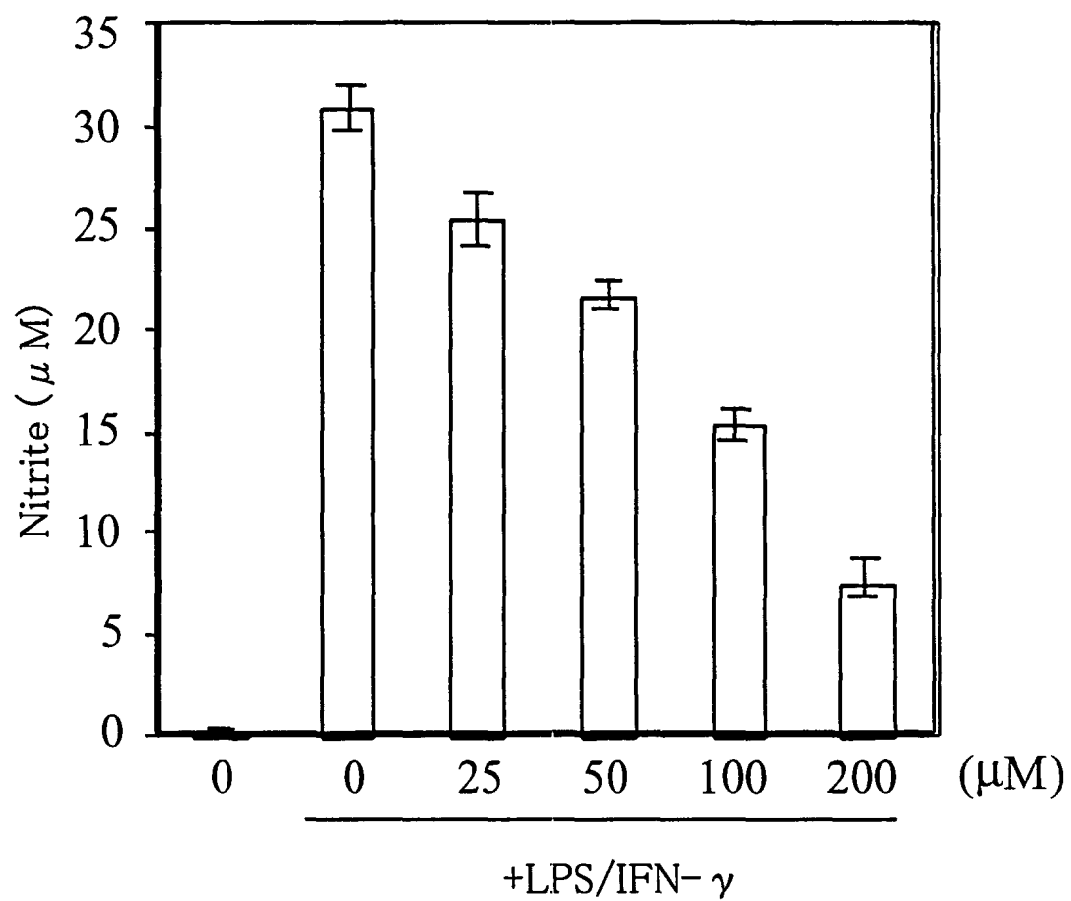   and α-tubulin protein

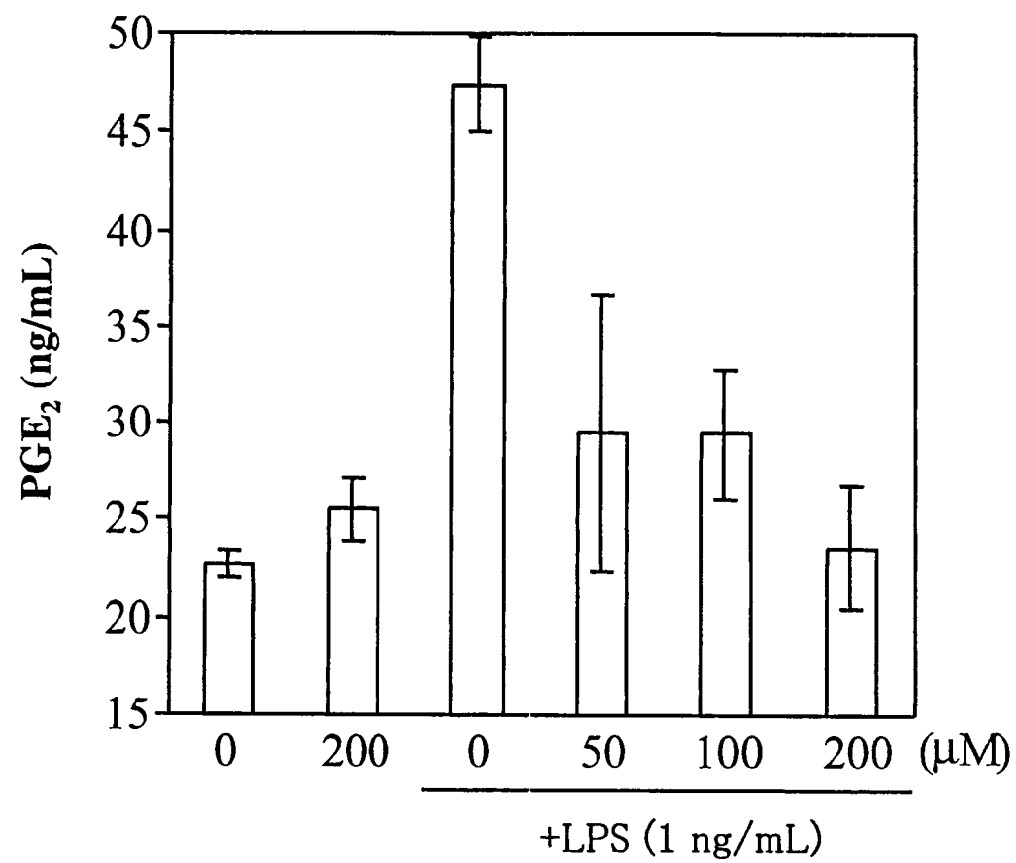Fig.2 Effects of agarobiose on LPS plus IFN-γ induced nitric production in RAW264.7 macrophages

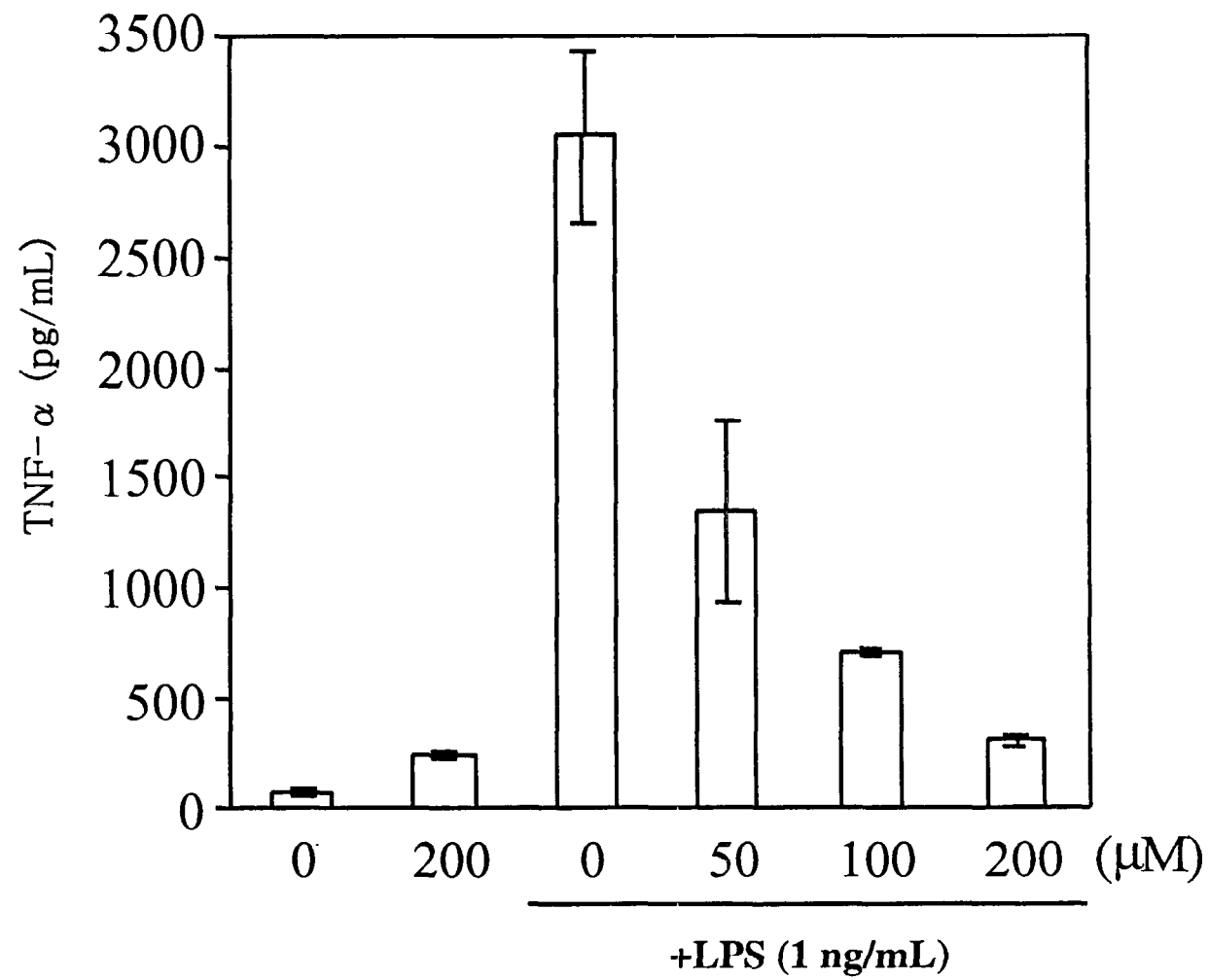Fig.3(a) Effects of agrobiose on LPS-induced PGE_2
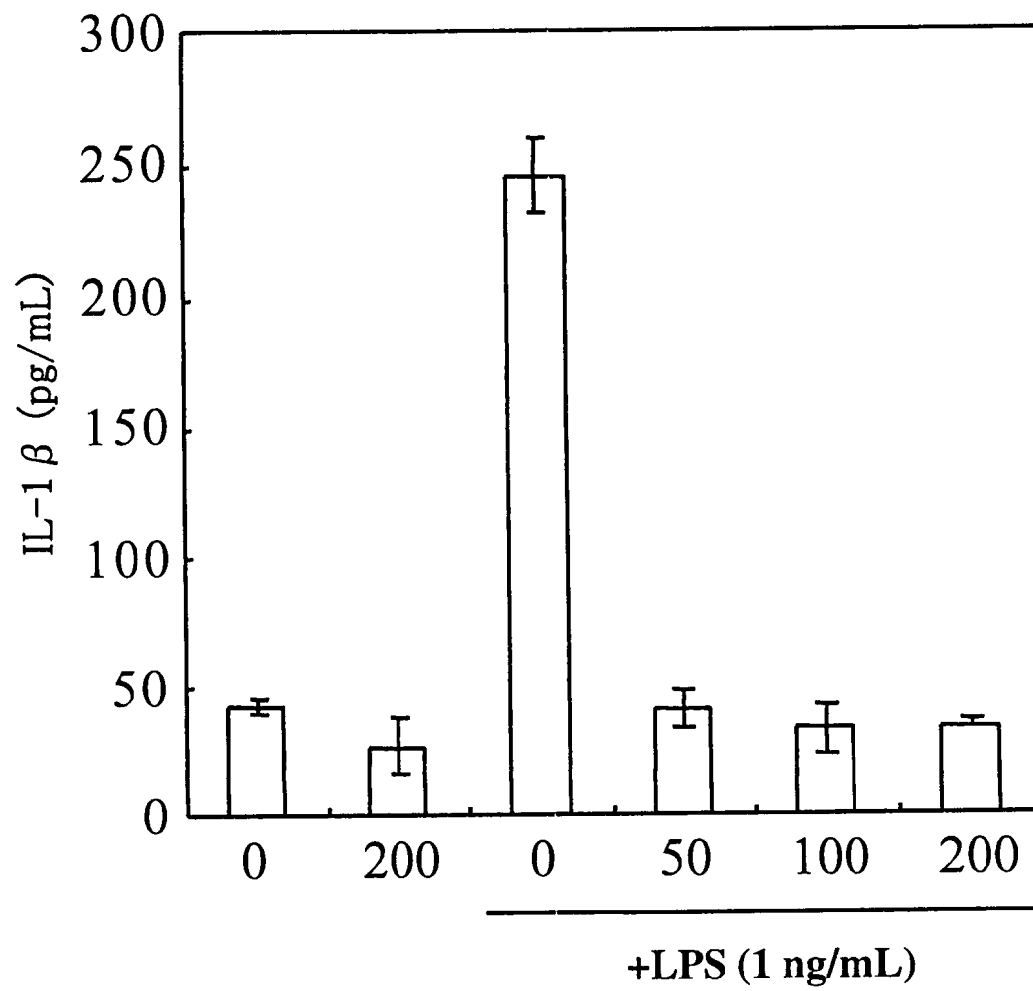
Fig.3(b) Effects of agarobiose on LPS-induced TNF-α

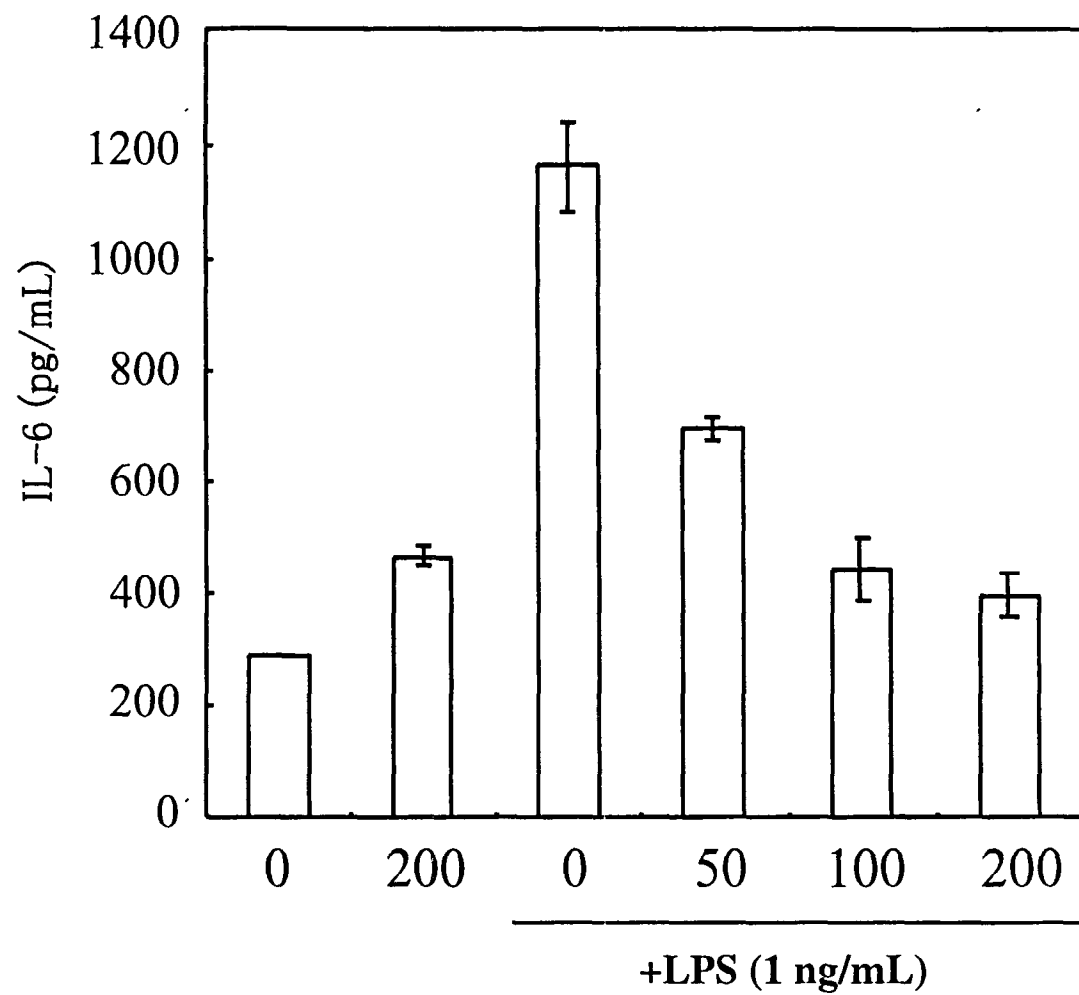Fig.3 (c) Effects of agarobiose on LPS-induced IL-1 β

Fig.3 (d) Effects of agarobiose on LPS-induced IL-6

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┌─────────────────────────────┐        ┌──────────────────────────────────────────────────────────┐
│  agaro-oligosaccharides     │        │  i) Inhibition of NO synthesis and elimination of activated oxygen  │
│                             │        │     (Inhibition of direct tissue injury)                    │
│            ↓                │        │                                                            │
│                             │        │  ii) Inhibition of PGE₂ production through specific inhibition of  │
│  Heme oxygenase-1 induction │   ⇒    │      COX-2 cascade                                          │
│                             │        │      (Anti-inflammatory effects with little adverse effects) │
│            ↓                │        │                                                            │
│                             │        │  iii) Inhibition of production of pro-inflammatory cytokines by CO  │
│  Production of CO, iron and │        │       (Inhibition of induction of inflammation)             │
│  bilirubin                  │        │                                                            │
└─────────────────────────────┘        └──────────────────────────────────────────────────────────┘
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Fig.4   Possible Site of Action of Agaro-oligosaccharides in the Prevation and Treatment of RA

9

Anti-inflammatory Effects of Agaro-Oligosaccharides

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Introduction

Kanten (agar), Iwanori, and Sushinori (Porphyra tenera) are representatives of red seaweed, which have been a food substance forming a key part of the everyday Japanese diet from ancient times. Over the last several years, the Biotech Research Laboratory of Takara Shuzo has conducted research on the functionality of agar, a representative of red seaweed[1]. The main component of the polysaccharides contained in agar is agarose, which is a linear sugar chain consisting of galactose (3-O-linked β-D-galactopyranose: abbreviated to "Gal") and anhydrogalactose (4-O-linked 3,6-anhydro-α-L-galactopyranose: abbreviated to "Ah-Gal") connected alternatively. Since the α1-3 linkage is unstable, agarose is degraded under slightly acidic conditions to produce agaro-oligosaccharides including agarobiose (consisting of Gal and Ah-Gal), agarotetraose (consisting of two pairs of agarobiose in tandem), and agarphexaose (consisting of three pairs of agarobiose). In other words, agarose is readily degraded by gastric juice to agaro-oligosaccharides, which are further converted to DGE, an active molecule, in the intestine[1]. In addition, agaro-oligosaccharides are very readily uptaken by hepatocytes because their nonreducing terminal is always Gal.

Many marvelous biological activities of agaro-oligosaccharides, which had never attracted special attention before, have been revealed in the course of our investigation. These activities include: the suppression of the overproduction of nitric monoxide (NO)[2], which is involved in the enhancement of the inflammatory response and may be considered to be one of the major subjects of research in modern medicine; enhancement of the production of carbon monoxide (CO)[3], which suppresses the inflammatory response; suppression of the production of TNF-a[4], which is deeply involved in the worsening of rheumatism. This article considers these activities in

more detail.

1. Production of nitric monoxide (NO) and physiological function

Surprisingly, nitric monoxide (NO), an air pollutant, plays an important role in the living body. This NO is synthesized by NO synthetase (NOS), using L-arginine as the starting material. It has been confirmed[5] by cloning that NOS has three isozymes, NOS-1, NOS-2, and NOS-3.

Of these, nNOS (neuronal NOS or NOS-1) is present in the cerebral and central nervous system, iNOS (Inducible NOS or NOS-2) in macrophages, and eNOS (endothelial NOS or NOS-3) in vascular endothelial cells. Especially, eNOS and nNOS are called calcium-dependent constitutive NOS, which constitutively produces NO free radicals for signal transmission in vascular and central nervous system cells and totally regulates these systems.

On the other hand, iNOS is produced by macrophages, and its expression is induced by cytokines and lipopolysaccharide (LPS). The produced NO protects the body by destroying foreign matters oxidatively and by regulating the cell growth in the blood and immune systems. In any case, iNOS is rarely expressed under a steady state, and is induced only when stimulated by cytokines and LPS[6].

However, overproduction of this inducible NOS (iNOS) occasionally destroys the vital function. For example, the damage to the dopaminergic neurons observed in Parkinson's disease is considered to occur when the iNOS level in brain glial cells increased for some reason, or when excessive NO is produced by iNOS in macrophages infiltrated into tissues in response to the cell damage[7]. In other words, it is considered that excessive NO reacts with superoxide extracellularly, forms peroxynitrite, causes damages to surrounding nerve cells, and finally destroys the nervous function. In addition, mad cow disease, which is feared around the world, is considered to be associated with NO and oxygen radicals[8].

It is known that the risk of developing atherosclerosis, which is a major cause of heart diseases, increases in inverse proportion to the blood concentration of high-density lipoprotein (HDL) cholesterol. However, the precise mechanism was not known until recently. Yuhanna et al. have discovered that HDL stimulates and activates the eNOS of vascular endothelial cells[9]. On the other hand, no activation of eNOS occurred with low-density lipoprotein (LDL). It was clarified that both eNOS and scavenger

receptor-BI (SR-BI) are co-localized in the specific sites of vascular endothelial cells and that eNOS is activated by the binding of HDL apolipoprotein (ApoA-I) to SR-BI.

2. Production of carbon monoxide (CO) and physiological function

(1) Production of anti-oxidant

In the living body, heme receives catabolism by heme oxygenase (HO) to yield equimolar quantities of carbon monoxide (CO), bilirubin, and iron. As in the case of NOS, HO has three isoforms. Of these isoforms, HO-2 and HO-3 are the constitutive type, and HO-1 is the inducible type, possessing a potent anti-inflammatory effect and also protecting the body from oxidative stress[10]. HO-1 is induced by heavy metals, various cytokines, hormones, endotoxins, heat shock, etc. In other words, HO-1 is strongly inducible by hydrogen peroxide, ultra violet, hyperoxemia, and other substances inducing oxidative stress. HO-1 is considered to maintain homeostasis by protecting cells and tissues from the damage induced by oxidants[11]. In rats in which the genes of HO-1 were introduced into the lungs using an adenovirus-derived vector, no damage was produced in the lungs even when the O_2 level was higher than 99%[12]. Therefore, the mechanism to protect cells from oxidative stress appears to be associated with the action of anti-oxidants such as bilirubin, ferritin, and CO, which is the major product from the catabolism of heme.

(2) Suppression of TNF-α production

It is not widely known that sepsis is a horrible disease for inpatients and is the principle cause of death. Death is caused not directly by the causative pathogens of the infection, but by the abrupt decrease in blood pressure due to the vasodilation caused by the induced NO, or by uncontrollable overproduction of TNF-α, IL-1β, macrophage inflammatory protein MIP-1β, and other proinflammatory cytokines and chemokines. Regarding the latter cause, such overproduction is known to cause increased white blood cells, leakage of peripheral blood vessels, and the destruction of tissues.

LPS, which is the main trigger of sepsis, is one of the cell wall components of Gram-negative bacteria. In mice (or macrophages) treated with LPS, severe symptoms such as those obtained in sepsis appear. However, anti-inflammatory cytokines such as IL-10 and IL-4 are produced some time later and exert their actions to alleviate the inflammation by inhibiting the synthesis of proinflammatory cytokines and chemokines.

When macrophage RAW264.7 cells are treated with LPS, the TNF-α production starts as expected. Choi et al. reported that TNF-α production in response to LPS was 5-fold higher in a macrophage cell line that does not express HO-1 than in another cell line that genetically expresses a large amount of recombinant HO-1[3]. This demonstrates that HO-1 significantly suppresses the TNF-α production.

Furthermore, it was found that the production of TNF-α decreases with the increasing concentration of CO when a macrophage cell line was treated with a trace amount of LPS in the presence of CO gas. In addition, in the presence of CO, the production of IL-1β and MIP-1β were also decreased. These results were also demonstrated in vivo experiments conducted in mice.

Next, Choi et al. investigated the LPS-induced TNF-α production in the presence/absence of CO in cells in which NO production was inhibited by the treatment with L-NAME, a NOS-specific inhibitor. They found that TNF-α production was not affected by L-NAME. In addition, CO was found to have no influence on LPS-induced NO production. These results have demonstrated that the suppression of TNF-α production is not the result of the effect of CO on the pathway involved in NO production.

3. Functionality of agaro-oligosaccharides

(1) Suppression of the inducible nitric monoxide synthetase (iNOS) expression

Our world first discovery is that, in mouse peritoneal macrophages treated with lipopolysaccharide (LPS) and interferon gamma (IFN-γ), the NO production increases due to the enhancement of iNOS expression, but in the presence of agarobiose the NO production decreases in inverse proportion to the amount of agarobiose (Fig. 1)[13]. Western blotting analysis using an anti-iNOS monoclonal antibody demonstrated that this decrease in NO production was caused by the decreased iNOS protein in response to agarobiose (Fig. 2). Next, we investigated the induced expression of iNOS mRNA following the pretreatment with agarobiose. The result showed stronger inhibition on the iNOS mRNA expression in proportion to the duration of agarobiose pretreatment (Fig. 3), demonstrating that agarobiose inhibits the iNOS mRNA expression.

Neoagarobiose, in which Gal and Ah-Gal are joined in the reverse manner to those of agarobiose, did not show any of those effects mentioned above.

(2) Suppression of prostaglandin E_2 production and TPA-induced edema

It has been confirmed that prostaglandin E_2 (PGE_2) is responsible for the following symptoms observed in joint inflammation: redness, swelling, hotness, and pain.

It was found that LPS-induced PGE_2 production during 18 hours post-induction was about 20% of the control level in the mouse macrophage cell line, RAW264.7, when the cells were pretreated for 5 hours with agarohexaose. According to the reigning theory, PGE_2 is normally produced from arachidonic acid by cyclooxygenase. However, when arachidonic acid was added, PGE_2 production was generally increased even in the presence of agaro-oligosaccharides, indicating that agaro-oligosaccharides do not inhibit cyclooxygenase. Therefore, it appears that agaro-oligosaccharides inhibit PGE_2 production through an unknown novel passway[14].

TPA, one of the phorbol esters, possesses an important effect that leads to tumor promotion by enhancing the cellular phospholipid metabolism. Therefore, suppressing the effect of TPA is regarded to be very important as an anti-tumor promoter. This tumor promoter TPA induces edema when applied to the ear in mice. It has been demonstrated that the oral treatment with a 10% agaro-oligosaccharide or its external application to the ear for 14 days prior to the application of TPA suppressed the induction of edema[15]. The effect of agaro-oligosaccharides was investigated in mice on papilloma induced by the administration of 100 µg DMBA, a carcinogenic substance, followed, from 1 week later, by treatment with 1 µg TPA applied over the dorsal skin twice weekly for a period of 20 weeks. Agaro-oligosaccharide solutions were given to those mice instead of drinking water, starting 1 week before the administration of DMBA. In those receiving the 3% agaro-oligosaccharide solution, the number of mice that developed papilloma was about 10% of the corresponding number obtained in the control group (receiving water) (Fig. 4).

(3) Expression of inducible carbon monoxide synthetase (HO-1)

Here, carbon monoxide synthetase means heme oxygenase. As mentioned earlier, heme undergoes catabolism by heme oxygenase (HO) and yields equimolar quantities of carbon monoxide (CO), bilirubin, and iron. The macrophage RAW264.7 cells did not express HO-1 after being cultured for 12 hours in a culture medium alone. Treatment of the cells with LPS alone or with LPS plus IFN-γ increased HO-1 expression slightly,

whereas the addition of agarobiose to the culture increased HO-1 expression in a concentration-dependent manner (Fig. 5)[16]. This is considered to be the result of the synergistic effect of agarobiose and the oxidative stress caused by LPS-induced NO. On the other hand, in addition to HO-1, other stress response proteins HSP-70 and GRP-78 were also investigated and found to be unaffected. This indicates that this effect is specific to NO and agarobiose.

(4) Suppression of TNF-α production

A study was carried out in mice that were allowed to have free access to an aqueous solution of heated agar as drinking water for 19 days. After 19 days, a high dose of LPS (300 μg/mouse) was given intraperitoneally to induce endotoxin shock. The mortality rate in the group treated with 1% agar solution was 100% (8/8 mice), whereas the rate in the group treated with 10% agar solution was 25% (2/8 mice). This clearly indicates the protective effect of agar against endotoxin shock. In the same manner, a low dose of LPS (20 μg/mouse) was given intraperitoneally to mice that had been treated with the agar solutions for 19 days. One hour after administration, the serum level of TNF-α was determined. In those treated with the 10% solution, the TNF-α-level was found to be lower by about 30%.

In another experiment, mouse macrophages were cultured in the presence of agarobiose for 6 hours, and then LPS was added to the culture to make the final concentration of 1 μg/mL. Twenty-four hours later, the amount of TNF-α produced was determined (Fig. 6)[17]. In the presence of 200 μM agarobiose, TNF-α production decreased by about 60%. These experiments were also carried out in monocytes derived from human peripheral blood mononuclear cells. In the human monocytes, the decrease in TNF-α production was observed with a lower concentration of agarobiose than in mice (Fig. 7).

4. Use as a supplement for preventing arthrosteitis

Health food supplements are available for various diseases. Chondroitin sulfate and glucosamine are available as supplements for arthrosteitis in the U.S. and held a 120-billion dollar market last year. The sole rationale for the indication that these compounds are effective against arthrosteitis is that these two compounds are the constituents of human joint cartilage. In the 1980s, it was reported that these

compounds relieve the pain associated with arthrosteitis, and the National Institute of Health (NIH) in the U.S. started a 4-year research project with a grant of about 7 million dollars.

As mentioned above, the oral administration of agaro-oligosaccharide derived from agar suppresses the expression of proinflammatory cytokines such as TNF-α, which is considered to be a cause of rheumatism, enhances the expression of heme oxygenase-1, and produces various molecules possessing anti-oxidative effects, such as carbon monoxide. In fact, it is said that a solidified agar solution is used habitually as a folk medicine effective against rheumatism in Peru and the surrounding countries in South America. In Tanabe Seiyaku Co., Ltd., a therapeutic drug for rheumatism (brand name: Remicade) is being developed from an anti-TNF-α antibody (in Phase II/III).

Taken together, it is highly likely to be demonstrated that agaro-oligosaccharides are effective against a certain type of rheumatism.

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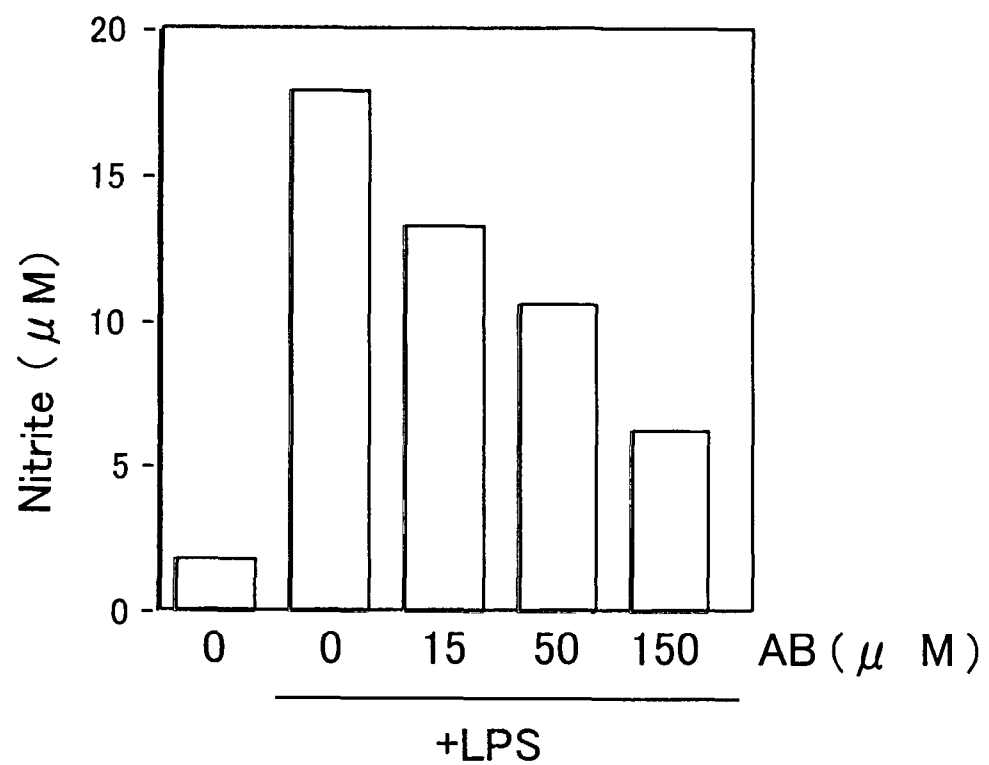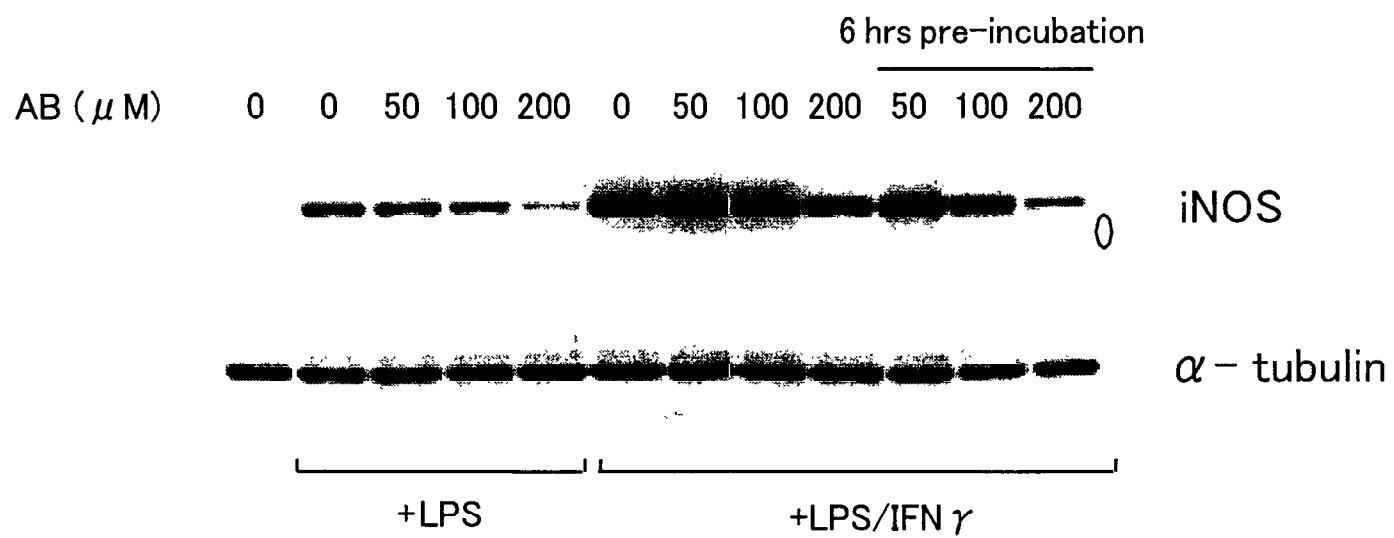
Fig.1 Effect of Agarobiose on NO production by mouse peritoneal macrophages

| AB (μM) | 0 | 0 | 50 | 100 | 200 | 0 | 50 | 100 | 200 | 6 hrs pre-incubation | | |
| | | | | | | | | | | 50 | 100 | 200 |

iNOS

α – tubulin

+LPS

+LPS/IFN γ
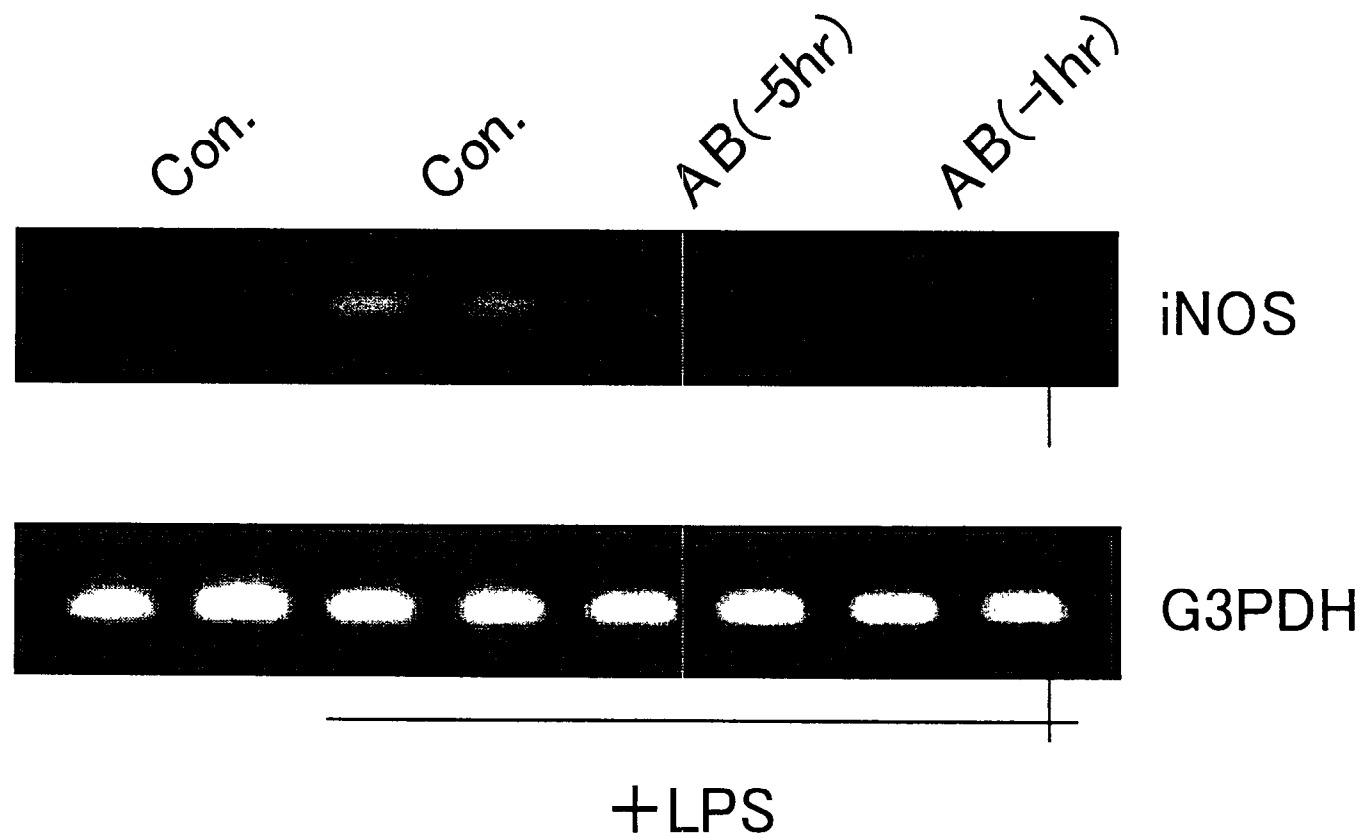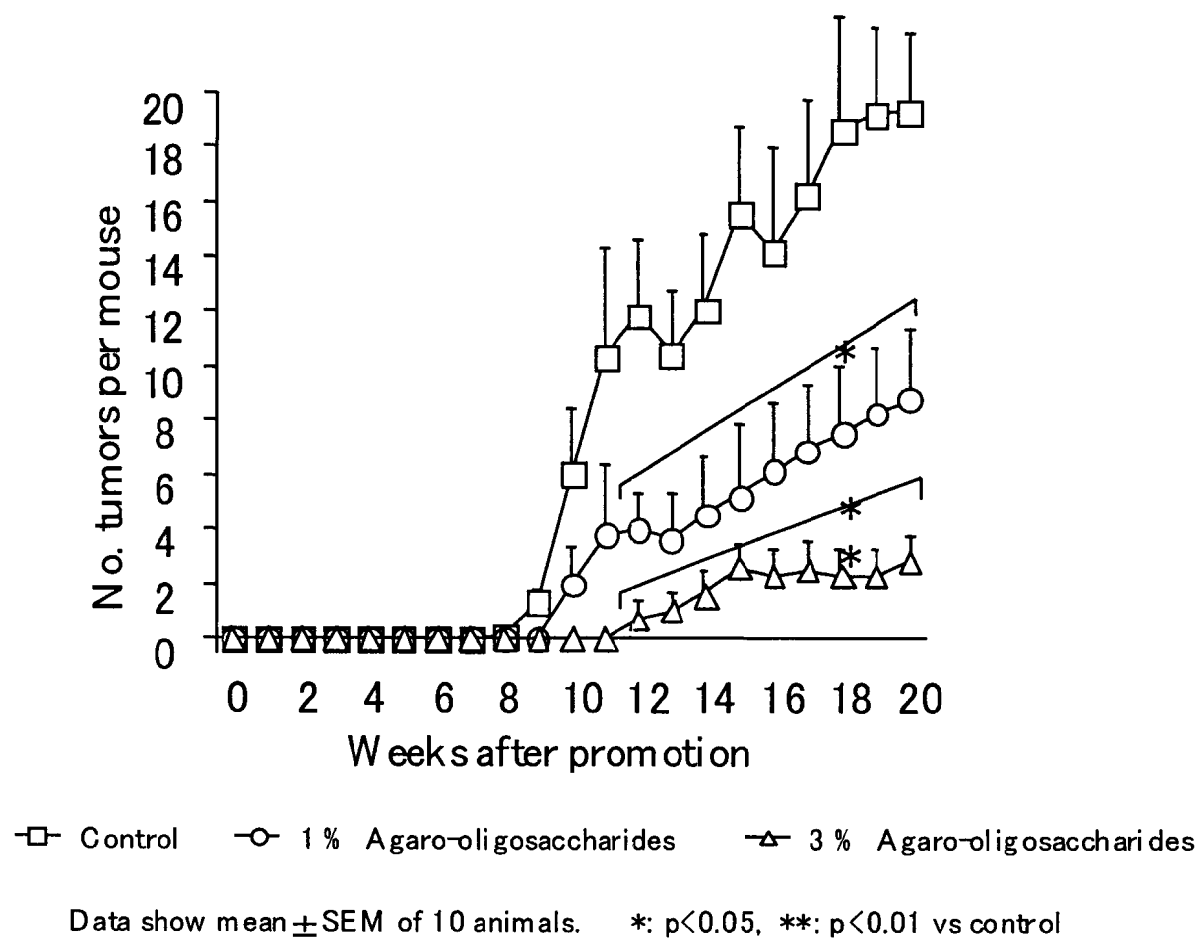
Fig.2 Suppression of iNOS protein expression by Agarobiose

Fig.3 Suppression of iNOS mRNA expression by Agarobiose

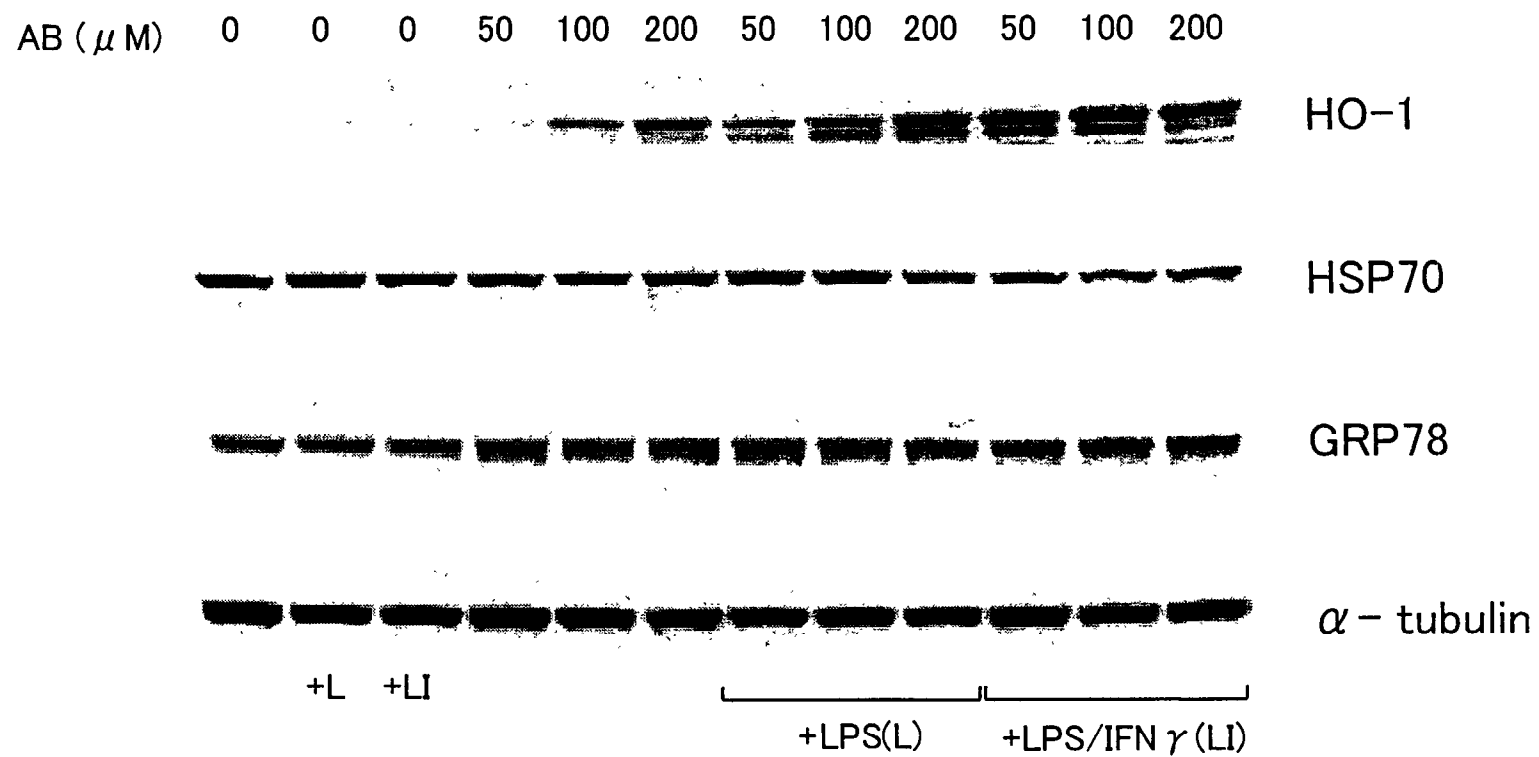Fig.4 Preventive effect of Agaro-oligosaccharide on cancer development

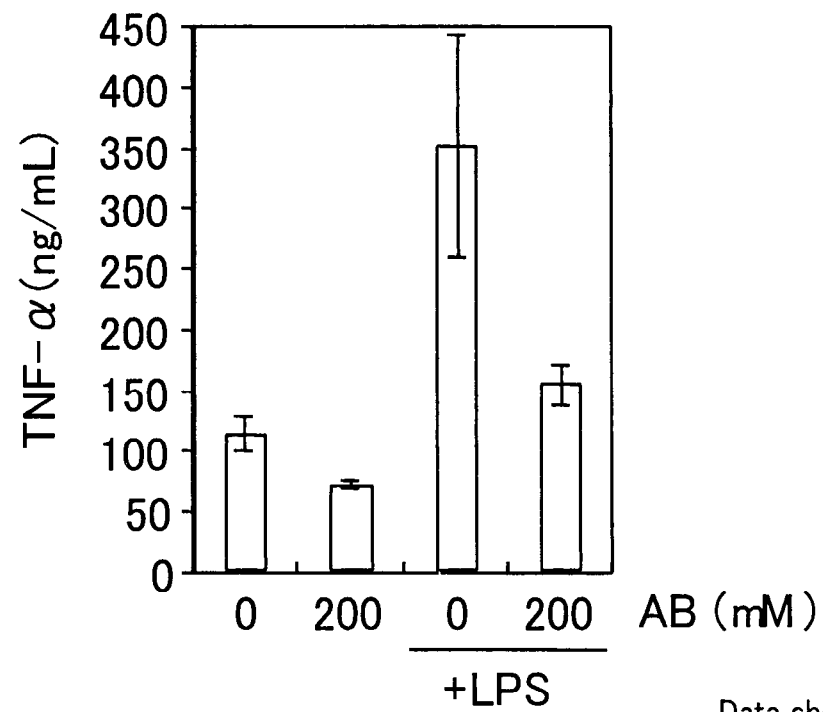Control   1 % Agaro-oligosaccharides   3 % Agaro-oligosaccharides

Data show mean ± SEM of 10 animals.   *: p<0.05, **: p<0.01 vs control

Fig.5 Induction of HO-1 expression by Agarobiose

Fig.6 Effect of Agarobiose on TNF-α production by mouse peritoneal macrophage
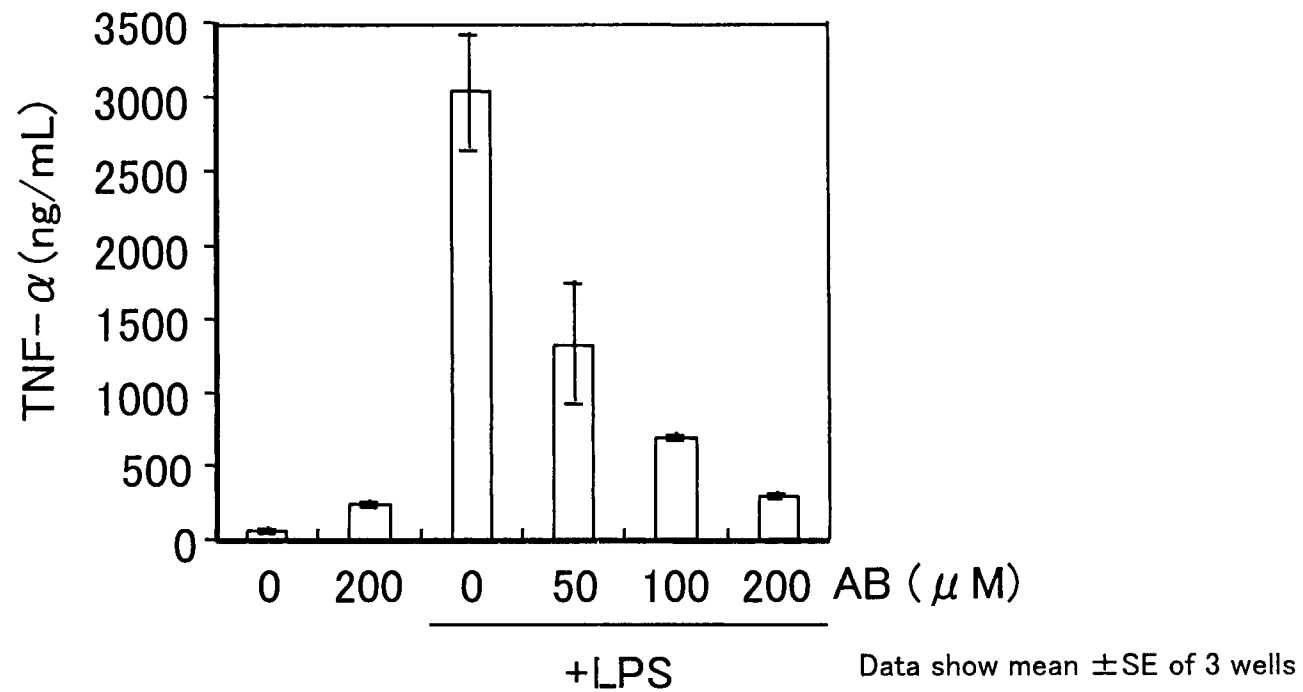
Data show mean $\pm$SE of 3 wells

Fig.7 Effect of Agarobiose on TNF-α production by the monocytes
derived from human peripheral blood mononuclear cells

Publication of Study on Agaro-oligosaccharide (1998-2000)

	Academic society	Period	Place	No	Subject	Speakers
1	The 20th Japanese Carbohydrate Symposium	1998.7.15-17	Sapporo	B1-05	Agaro-oligosaccharides induce apoptosis in HL-60 cells and suppress nitric oxide production in macrophage	Tatsuji Enoki, Shinji Okuda, Hiroaki Sagawa, Katsushige Ikai and Ikunoshin Kato
2	The 57th Annual Meeting of the Japanese Cancer Association	1998.9.30-10.2	Yokohama	2232	Effect of Agaro-oligosaccharides from red algae on proliferation and nitric oxide production in mammalian cell lines	Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
3	The 71th Meeting of the Japanese Biochemical Society	1998.10.14-17	Nagoya	3N-W29-06	Agaro-oligosaccharides induce apoptosis in HL-60 cells and suppress nitric oxide production in macrophage	Tatsuji Enoki, Shinji Okuda, Hiroaki Sagawa, Katsushige Ikai and Ikunoshin Kato
4	The 6th Japanese Society for Cancer Prevention	1999.7.16-17	Tokyo	P-37	Basic studies on Agaro-oligosaccharides as cancer-preventive food product	Tatsuji Enoki, Shinji Okuda, Takanari Tominaga, Hiroaki Sagawa and Ikunoshin Kato
5	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29-10.1	Hiroshima	246	Inhibitory effects of Agaro-oligosaccharides on NO and PGE_2 generation in activated-macrophages	Tatsuji Enoki, Takanari Tominaga, Hiroaki Sagawa and Ikunoshin Kato
6	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29-10.1	Hiroshima	247	Effect of Agaro-oligosaccharides analogs on proliferation and NO synthesis in mammalian cells lines	Eiji Kobayashi, Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
7	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29-10.1	Hiroshima	248	Anti-inflammatory action of Agaro-oligosaccharides on TPA-induced inflammation in mice	Takanari Tominaga, Eiji Nishiyama, Tasuji Enoki, Hiroaki Sagawa, Shigetoshi Mizutani and Ikunoshin Kato
8	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29-10.1	Hiroshima	2258	Anti-tumor activity of Agaro-oligosaccharides from agar against human colon cancer xenografts	YU FU-gong, Takeshi Sakai and Ikunoshin Kato
9	The 72th Meeting of the Japanese Biochemical Society	1999.10.6-9	Yokohama	2P-001	Preventive and therapeutic effect of Agaro-oligosaccharides in acute and chronic inflammation models	Eiji Nishiyama, Suzu Deguchi, Kinya Fujii, Hiroaki Sagawa, Shigetoshi Mizutani and Ikunoshin Kato
10	The 72th Meeting of the Japanese Biochemical Society	1999.10.6-9	Yokohama	2P-115	Novel α-agarose derived from seaweed and microorganisms	Jun Tomono, Keiko Nomura, Hiroaki Sagawa, Takeshi Sakai and Ikunoshin Kato
11	The Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	3D040 β	Novel α-agarose produced by marine microorganisms	Jun Tomono, Keiko Nomura, Takeshi Sakai, Hiroaki Sagawa and Ikunoshin Kato

Publication of Study on Agaro-oligosaccharide (1998-2000)

	Academic society	Period	Place	No	Subject	Speakers
12	The Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	2E043 β	Correlation between induction of HO-1 and suppression of nitric oxide production by Agaro-oligosaccharides	Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
13	The 59th Annual Meeting of the Japanese Cancer Association	2000.10.4-6	Yokohama	1927	Correlation between induction of HO-1 and various physiological activity by Agaro-ologosaccharides, and analysis of gene expression with DNA-chip technology	Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
14	Japanese Association for Dietary Fiber Reseach, 5th Reseach Meeting	2000.11.17-18	Tokyo	4	Polysaccharides from Seaweeds : Agarose and Fucoidans as Functional Foods	Ikunoshin Kato